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Growth kinetics and transplantation of human retinal progenitor cells

Unber Aftab [a,*], Caihui Jiang [a], Budd Tucker [a], Ji Yeon Kim [a], Henry Klassen [b], Erik Miljan [c], John Sinden [c], Michael Young [a]

[a] Schepens Eye Research Institute, Harvard Medical School, 20 Staniford Street, Boston, MA 02114, USA
[b] Gavin Herbert Eye Institute, Department of Ophthalmology, School of Medicine, University of California, Irvine, 101 The City Drive, Bldg. 55, Room 204, Orange, CA 92868-4380, USA
[c] ReNeuron Ltd., 10 Nugent Road, Surrey Research Park, Guildford, Surrey, GU2 7AF, UK

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ABSTRACT

We studied the growth kinetics of human retinal progenitor cells (hRPCs) isolated from donor tissue of different gestational ages (G.A.), determined whether hRPCs can be differentiated into mature photoreceptors and assessed their ability to integrate with degenerating host retina upon transplantation. Eyes (12–18 weeks G.A.) were obtained with IRB approval and retinas were enzymatically dissociated. Cells were expanded in vitro, counted at isolation and at each passage, and characterized using immunocytochemistry and PCR. GFP positive hRPCs were co-cultured with retinal explants from rd1 and rhodopsin −/− mice, or transplanted into B6 mice with retinal photocoagulation and rhodopsin −/− mice. Eyes were harvested for histological evaluation following transplantation. Our results show that hRPCs from 16 to 18 weeks G.A. had the longest survival in vitro and yielded the maximum number of cells, proliferating over at least 6 passages. These cells expressed the retinal stem cell markers nestin, Ki-67, PAX6 and Lhx2, and stained positively for photoreceptor markers upon differentiation with serum. Some of the GFP positive cells used for transplantation studies showed evidence of migration into the degenerative host retina and expressed rhodopsin. In conclusion, we have determined the growth kinetics of hRPCs and have shown that cells from donor tissue of 16–18 weeks G.A. exhibit the best proliferative dynamics under the specified conditions, and that hRPCs can also be differentiated along the photoreceptor lineage. Further, we have also demonstrated that following transplantation, some of these cells integrate within the host retina and differentiate to express rhodopsin, thereby supporting the potential utility of hRPC transplantation in the setting of retinal degenerative disorders.

1. Introduction

The human retina is subject to a wide range of diseases that lead to the loss of photoreceptors and other retinal neurons resulting in permanent visual deficits. These include hereditary degenerations such as retinitis pigmentosa, as well as more widespread conditions such as macular degeneration, retinal detachment and diabetic retinopathy. Because of the lack of effective self-repair in the human retina, there is a pressing clinical need for novel therapeutic approaches to the treatment of retinal degeneration. One proposed method of treatment is retinal replacement through the use of an indwelling photosensitive electronic prosthesis (Chow et al., 2004; Colodetti et al., 2007; Loewenstein et al., 2004; Schanze et al., 2007). Fundamental problems with information transfer at the bioelectronic interface have thus far limited the success of this technology. An alternate approach to retinal 'chip', as it is known, involves cellular reconstruction of the neural retina using cells that are inherently predisposed towards a retinal cell fate, such as the progenitor cells of the developing neural retina.

In more recent work, we and others have derived neural retinal progenitor cells (RPCs) from a range of mammalian species, including mouse (Klassen et al., 2004a; MacLaren et al., 2006), rat (Chacko et al., 2000; Yang et al., 2006, 2002a), pig (Klassen et al., 2007) and human (Carter et al., 2007; Kelley et al., 1995; Klassen et al., 2004b; Yang et al., 2002b). Work in the rodent has shown that RPCs can be propagated extensively, express immature markers in culture, as well as mature photoreceptor markers following transplantation to the subretinal space of allorecipients and are associated with behavioral benefits in retinal dystrophic recipients

* Corresponding author. Tel.: +1 617 912 7421 (office); fax: +1 617 912 0101. Cell: +1 425 283 3479.
E-mail addresses: unber.aftab@schepens.harvard.edu (U. Aftab), caihui.jiang@schepens.harvard.edu (C. Jiang), budd.tucker@schepens.harvard.edu (B. Tucker), ji.kim@schepens.harvard.edu (J.Y. Kim), hklassen@uci.edu (H. Klassen), erik-miljan@reneuron.com (E. Miljan), john-sinden@reneuron.com (J. Sinden), michael.young@schepens.harvard.edu (M. Young).
URL: http://www.schepens.harvard.edu

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(Klassen et al., 2004a; MacLaren et al., 2006). We next extended this work to a pig allograft model and have been able to reproduce many of the results seen in mice (Klassen et al., 2007). Replication of such findings with the transplantation of human RPCs will be a breakthrough in the field of retinal transplantation as a treatment strategy for degenerative retinal disorders.

Progenitor cells have in fact been grown from the neural retina of humans (Carter et al., 2007; Kelley et al., 1995; Klassen et al., 2004b; Yang et al., 2002b). However, a major challenge that has emerged with respect to the use of hRPCs is the limited ability of these cells to expand in culture. Little is known about their proliferative dynamics and their behavior following transplantation has not been studied thus far. The work performed in the current study was primarily directed towards understanding the growth kinetics of hRPCs while comparing different gestational ages. The study also focuses on assessing whether hRPCs can generate cells with rod and cone photoreceptor phenotypes.

In addition, it was of great interest to evaluate the behavior of these cells within the retinal microenvironment of explant and transplantation models. We assessed whether these cells can migrate and integrate with the host retina and differentiate into mature photoreceptors once transplanted into degenerative recipient tissue.

2. Materials and methods

2.1. Isolation and cell culture

Human fetal eyes [12–18 weeks gestational age (G.A.)] were obtained through therapeutic termination of pregnancy from Advanced Bioscience Resources with informed consent and IRB approval. Whole neuroretinas from each eye were dissected out and minced into small pieces as previously described (Klassen et al., 2004a,b, 2007). The tissue was dissociated by spinning at 250 rpm for 20 min using 0.1% collagenase in DMEM-F12. Dissociated cells were then transferred to a 15 ml tube and the undissociated tissue was re-suspended in fresh 0.1% collagenase and spun through successive cycles – a total of four cycles were performed. The dissociated cells were centrifuged at 850 rpm for 3 min at room temperature and the pellet was re-suspended in Rencell media (Chemicon) supplemented with 5% Fetal Bovine Serum (FBS), 20 ng/ml Epidermal Growth Factor, 10 ng/ml basic Fibroblastic Growth Factor, 2 nM L-glutamine and 50 μg/ml Gentamycin. Aggregates of cells were dissociated through gentle trituration and the number of live and dead cells was counted through a trypan blue assay.

Cells were plated in fibronectin (100 μg/ml) coated tissue culture flasks at a seeding density of 9000–13 000 cells/cm^2 and were incubated at 37 °C and 5% CO_2. In order to increase cell survival and the plating efficiency following harvest, cells were kept in media supplemented with 5% FBS during the first day of isolation. A complete media change with supplemented Rencell media without FBS was performed after 24 h and was used throughout subsequent cell culture and expansion.

Cells were fed every alternate day by replacing half the volume with fresh media. Cells were passaged when they reached 70–85% confluence, (usually within 3–5 days). Briefly, cells were rinsed with HBSS and dissociated with 0.25% trypsin. The cells were then rinsed with trypsin inhibitor solution in DMEM-F12 and centrifuged at 850 rpm for 3 min. The pellet was re-suspended in supplemented Rencell media, cells were counted at each passage and were plated into new fibronectin coated flasks at the seeding density mentioned above. Using these cell counts, growth was monitored for each donation over several passages.

We used fibronectin for coating purposes throughout cell culture. However, we observed differences in adhesion properties and morphology between cells grown in freshly treated fibronectin and pre-coated laminin plates, and hence compared the two substrates. For experimental purposes only, hRPCs were plated into either laminin or fibronectin (100 μg/ml) coated flasks at a constant seeding density and their morphology was assessed. Also, in order to confirm whether fibronectin was essential as a substrate for an adherent culture, 96 well plate experiments were set up with cells being plated onto either fibronectin coated plates or non-coated plates (Costar). Cell counts were obtained on days 1, 3 and 6 using CyQuant Assay Kit (Invitrogen) and a microplate reader, and the results were statistically analyzed using a paired *t*-test.

2.2. Immunocytochemistry and PCR

4000 cells were plated in each well of fibronectin coated (100 μg/ml) 16-well chamber slides at the time of isolation and passage. Cells were either differentiated with 5% FBS in supplemented Rencell without mitogens over 7 days or left in standard undifferentiated conditions. Cells were fixed on day 7 with 4% paraformaldehyde, blocked with 10% goat serum in PBS for 1 h and incubated with antibodies against Ki-67 (Vector 1:100), nestin (Chemicon 1:200), rhodopsin (Chemicon 1:100), recoverin (Chemicon 1:1000), red/green opsin (Chemicon 1:300) and blue opsin (Chemicon 1:300) overnight at 4 °C. Cells were then rinsed and incubated with the corresponding Cy3 secondary antibodies (Chemicon 1:200) at room temperature for an hour. Slides were again rinsed after incubation and mounted with Vectashield mounting agent with DAPI (Vector Labs) and observed under a Nikon fluorescence microscope. The expression of markers studied was compared between the undifferentiated and differentiated cells.

For PCR experiments, total RNA from 2 to 4 million undifferentiated early and late passage (P3 and P6) hRPCs grown on fibronectin was extracted using the RNeasy Mini-kit following the manual instructions. Due to the availability of a single cell population for passage three, we were restricted to the use of only one sample for that passage. Cells were lysed, homogenized and ethanol was added to adjust binding conditions. Samples were spun using spin-columns, washed and eluted using RNase-free water. 1 μg of RNA was reverse transcribed into cDNA using the random hexamer (Invitrogen, Grand Island, NY) priming method and Omniscript reverse transcriptase (Qiagen, Valencia, CA). All PCRs were performed in a 40 μL reaction containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 100 ng of DNA, 1.0 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA) and 20 pmol of each gene specific primer (Banin et al., 2006; Bibb et al., 2001; Lamba et al., 2006). All cycling profiles incorporated an initial denaturation temperature of 94 F for 10 min, 35 amplification cycles (30 s at 94 F, 30 s at annealing temperature of each primer and 1 min at 72 F) and a final extension at 72 F for 10 min. PCR products were electrophoretically separated on 2% agarose gels. The genes studied include various retinal proteins and transcription factors: *PAX6*, *LHX2*, *CRX*, *ATOH7* (math5), *RCVRN* (recoverin), *RHO* (rhodopsin) and *OPN1SW* (blue opsin). PCR reagent without c-DNA was used as a negative control.

2.3. Retroviral infections

MMLV type retrovirus encoding the GFP transgene in the pLNCX vector under the human cytomegalovirus (CMV) immediate early promoter was generated using the TEFLY-A (amphotropic) virus packaging cell line as previously described (Cosset et al., 1995). Since the virus infects actively dividing cells, we used early passage (P1–P2) hRPCs from donor tissue of 17–18 weeks G.A. – these cells are found in a logarithmic phase representing active cell proliferation and exhibit maximum growth rates. hRPCs were transfected with the GFP gene through retroviral infections over a period of 3